Manganese neurotoxicity: a model for oxidative stress and selective neurodegeneration in the basal ganglia

Intoxications and other pathologies of the CNS are most often characterized by selective damage. In particular, the basal ganglia that control automatic movements are vulnerable brain regions under abnormal conditions such as idiopathic Parkinson's disease (iPD) and manganese (Mn) poisoning. At present the etiology and pathogenesis of iPD (and related disorders) as well as the mechanism of Mn-induced neurodegeneration of the basal ganglia (or most other intoxications affecting this area) are unkown. In addition, both pathologies result in irreversible hypokinesia (rigidity, tremor, bradykinesia, unstable postural reflexes, dystonia), while there is no long-lasting effective treatment available.

Specific properties, or a unique combination of basic, endogenous factors of the basal ganglia, such as the presence of dopamine (DA), glutamate (Glu), iron (Fe) and neuromelanin, may render them more susceptible to damage, in particularly oxidative injury, than other brain regions. Since all these intrinsic factors can participate in oxidative stress events via different mechanisms (see Chapter 1), which are often considered a final common pathway of cell death, this may explain the selective vulnerability of the basal ganglia under abnormal conditions. On the other hand, defects in generally occurring cell systems, including the mitochondrial respiration chain (the main natural source of reactive oxygen species) and oxidative defense mechanisms such as glutathione (GSH) metabolism, may lead to selective oxidative damage of the basal ganglia because of their intrinsic metabolic properties. Questions addressed to understand this vulnerability may provide better fundamental and therapeutic insight for the treatment of basal ganglia disorders.

One should bear in mind that there exists no 'perfect' animal model for PD as it occurs in humans. All models of Parkinsonism show differences and similarities with iPD, of which Mn intoxication may be specified as a model for dystonia. Table 1 summarizes various oxidative stress phenomena in relation to basal ganglia lesions which cause hypokinetic movements as outlined in Chapter 1, including Mn-induced lesions which are described in this thesis. In the present thesis, Mn-neurotoxicity has been chosen as a model to study *in vivo* fundamental aspects of oxidative stress and selective neurodegeneration in the rat basal ganglia. In particular, the role of iron and DA in producing

oxidative stress by Mn is emphasized as a relevant and integral part of Mn neurotoxicity and one of the main topics in current PD research.

Using systemic Mn administration to study its neurotoxicity has the practical disadvantage, that Mn accumulates (very) slowly in the brain and that the onset of lesions in the basal ganglia will not occur until several months after chronic exposure. Since some authors have reported acute depletions of DA after a single Mn injection into rat striatum or substantia nigra, we used this experimental approach to further characterize an acute Mn intoxication model in teh rat. It followed from our studies that intrastriatal Mn injection produced time- and dose-dependent DA depletions and displayed selective nerve cell death as reflected in ^{45}Ca accumulation in the basal ganglia, which virtually matched the described pathology after chronic systemic exposure to Mn. The observed regional distribution of ^{45}Ca accumulation in striatum, globus pallidus (GP), entopeduncular nucleus (external GP), substantia nigra and several thalamic nuclei involved in motor functions, suggested both pre- and post-synaptic damage to the DAergic nigrostriatal pathway. Postsynaptic damage probably includes GABAergic neurons.

Selective effects of Mn were also observed at the level of biogenic amines in striatum and substantia nigra: partially reversible DA depletions, and reversible changes of noradrenaline (NE) and serotonin neurotransmitter levels in striatum. In addition, DA neurotransmission in striatum, based on measurements of the 'synaptic' DA-metabolite 3-methoxytyramine, was completely abolished at day 1 and 3 after Mn injection, but recovered within 6 weeks. This indicated a functional restoration of DA neurotransmission, despite still substantial losses of tissue DA, and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA).

Selectivity of both Mn and Fe was further tested by comparing injections of either metal in striatum or hippocampus, two areas with a respectively high and low DA and iron content. These studies demonstrated a relatively low toxicity of Mn in the hippocampus, and a high toxicity of Fe in the brain independent of the injection site. The finding that the gyrus dentatus, a subregion of the hippocampus with the highest iron levels locally (albeit still low compared to the basal ganglia), was the preferential site for ^{45}Ca accumulation after intrahippocampal Mn-injection, was consistent with our hypothesis that Mn needs iron or iron-binding sites to exert its toxicity. In addition, the selective lesions by Mn and extensive damage by Fe after intracerebral injection suggest different mechanisms of action.

Characterized in more detail, this Mn model offered the possibility to further study the mechanism or prevention of Mn neurotoxicity with the use

of pharmaceutical intervention (antioxidants, DA/Glu (ant)agonists, etc.). With this in mind, the distribution, retention and transport of Mn was studied, which could possibly help to explain some aspects of its selective toxicity. Using trace injections of ^{54}Mn into striatum or substantia nigra, it was not only demonstrated that substantial amounts of label were retained in both regions until at least 2-3 days after injection, but also that Mn was transported by neurons. By blocking axonal transport through the medial forebrain bundle using a local colchicine injection or mechanical transection of the bundle, or by producing selective lesions of the DAergic nigrostriatal pathway (using 6-OHDA) or GABAergic striatonigral pathway (using quinolinic acid), it was shown that Mn is transported through nigro-striatal and striato-nigral nerve fibres in anterograde direction.

In addition, the striatum lesioned with quinolinic acid, which is depleted of most intrinsic nerve cells and contains reactive glial cells, accumulated three times more ^{54}Mn, suggesting a predominant micro- and/or astroglial localization of ^{54}Mn. The exact cellular entity responsible for Mn transport is not known from these or other studies, but it is discussed that iron transport pathways and storage proteins are the most likely candidates that may determine selective transport and accumulation of Mn in and within the basal ganglia.

Since it has been hypothesized from *in vitro* studies, that Mn may produce free oxygen radicals directly or indirectly via auto-oxidation of catecholamines, our next goal was to measure free oxygen radicals *in vivo* in direct relation to (biochemical) damage. Recently, salicylate (SA) has been used as an *in vivo* trapping agent for the highly deleterious hydroxyl radicals ($^{\cdot}$OH), which upon reaction with SA form the relatively stable adducts 2,3- and 2,5-dihydroxybenzoic acids (DHBA). For that purpose, we developed a HPLC method to analyze catechol- and indoleamines and related metabolites in combination with SA and its hydroxylated adducts 2,3- and 2,5-DHBA (in striatum and CSF). This enabled us to study metal-induced $^{\cdot}$OH formation and DA/5-HT changes simultaneously in the same tissue samples. Since iron is considered to catalyze the Haber-Weiss reaction *in vivo*, thereby producing $^{\cdot}$OH, it was obvious to study brain Fe intoxication using this $^{\cdot}$OH-trapping technique. In addition, the contrast between a hypothesized different mechanism of action of both transition metals (as concluded above), and their close physico-chemical interrelationship and thus possible interference of Mn with brain iron homeostasis, may shed light on aspects of Mn neurotoxicity and oxidative stress. In other words, endogenous iron released by Mn may be the crucial factor in Mn-toxicity.

Time-course studies expanding from 30 minutes to 1 month following intrastriatal Fe injection revealed that 2,3-DHBA, the non-enzymatic product from SA and ·OH, peaked at 30 minutes (10-fold increase), whereas DA and 5-HT levels were relatively unaltered during the first two hours. These data suggest, that Fe-triggered ·OH formation precedes striatal DA (which was irreversibly depleted) and 5-HT depletions which occurred at later time points (beyond 2 hours). In addition, ·OH are continuously formed during Fe-induced neurodegeneration in a gradually declining fashion. At two hours after Fe injection, the high DOPAC level, no change in HVA, and a moderately reduced DA level, indicated specific enhancement of both DA anabolism and catabolism. Based on the iron dependency of the rate-limiting DA-synthetic enzyme tyrosine hydroxylase (TH), and the specific localization of TH (cytosol) and the DA catabolic enzymes monoamine oxidase (outer membrane of mitochondria) and catechol-*o*-methyltransferase (extraneuronally), it was concluded that this Fe-effect was within the DA neuron.

Whether the SA ·OH-trapping technique could be applied in the clinic to study chronic conditions of oxidative stress, for example by measuring SA adducts in CSF of PD or amyotrophic lateral sclerosis (ALS) patients, was experimentally investigated in CSF of rats injected with Fe into striatum. Indeed, DHBA levels in CSF of these rats were significantly elevated at time-points 30 minutes and 3 days. However, less severe "lesions" produced by Mn in striatum (as examined until 18 h; see Chapter 6) did not induce significantly elevated DHBA levels in CSF. Whether chronic neurodegenerative diseases may produce increased DHBA levels in CSF remains to be resolved. Since comparable doses of SA (100 mg/kg) are used to treat juvenile arthritis, the relatively high SA dose needed for such studies in humans is still in the clinically safe range.

Subsequent studies described in Chapter 6 showed that intrastriatal Mn injections increase 2,3-DHBA formation in a time- and dose-dependent manner in striatum, suggesting that Mn induces ·OH *in vivo*. Since the 2,3-DHBA effects by Mn demonstrated a time-delayed increase (maximum at 6-18 h), in contrast to our 2,3-DHBA effect by Fe over time, it was concluded that Mn induces ·OH by an indirect mechanism, implicating that Mn can not catalyze the Haber-Weiss reaction *in vivo*. It also followed from time- and dose-related effects (of simultaneously measured DA and 2,3-DHBA) that DA depletion and ·OH formation by Mn appear to be independent processes. It has been suggested by *in vitro* observations that Mn oxidizes DA rapidly and irreversibly to its cyclized *o*-DA-quinone resulting in a decrease of DA, but not in the formation of reactive oxygen species, since oxygen is neither consumed nor required. This is in line with the rapid initial DA-depleting effect induced by Mn

in vivo without a significant rise of ·OH in the first two hours, indicating that Mn-induced DA depletion *in vivo* is similar to that observed *in vitro*. However, the reducing capacity of DA neurons will probably be exhausted by such DA (auto)oxidation processes (and together with mitochondrial energy depletion) resulting in, for example, a severely compromised GSH metabolism as has been shown by Liccione and Maines, which could make these cells potentially more susceptible (e.g. to excitotoxicity).

In an attempt to find out via what mechanism ·OH are formed by Mn we investigated the possible role of DA (or non-enzymatic DA (auto)oxidation products) and 'free' or low-molecular-weight (LMW) iron as a potential source that can catalyze the Haber-Weiss reaction. Studies in which rats were depleted of striatal DA (90% reduction) by pre-treatment with reserpine, or in which rats received co-injections of Mn and the iron chelator deferoxamine (DFX) into striatum, did not demonstrate attenuation of Mn-induced 2,3-DHBA formation, while the extent of DA reductions was similar. Furthermore, the level of chelatable LMW iron in Mn-injected striata was not different from controls. In conclusion, these findings suggest that neither DA or DA (auto)oxidation products nor chelatable iron participate in Mn-induced ·OH formation.

Other possibilities by which the observed Mn-induced ·OH formation might be explained are (1) through the formation of peroxynitrite (ONOOH), the reaction product of NO· and $O_2^{\cdot-}$, or (2) heme-associated ferryl radicals. The former implicates iron-independent ·OH formation from the decomposition of peroxynitrite. Since Mn shares aspects of (additional) excitotoxicity with several other mitochondrial toxins whose neurotoxicity could be attenuated by NO-synthase inhibitors, NO-triggered oxidative damage after Mn might be a possible mechanism. Other features of Mn toxicity, such as impairment of (mitochondrial) GSH metabolism and several mitochondrial energy supply functions by Mn, including heme-associated functions (respiratory and P-450 cytochromes) and oxidative phosphorylation, may lead to or be the result of formation of heme-associated ferryl radicals, which are similar in reactivity to ·OH or ferryl iron (FeO^{2+}). If the postulated formation of heme-associated ferryl radicals (and H_2O_2) occurs, this would imply that such radicals are capable of hydroxylating SA, which is not unlikely and could be tested *in vitro*.

In summary, it is proposed that the selective neurotoxicity of Mn within the basal ganglia is most likely exerted via iron-dependent distribution characteristics (transferrin receptors, ferritin) and subsequent transport (Ca uniporter) that determine the preferential accumulation into mitochondria. Here, high local Mn concentrations will disturb several important energy supply functions and anti-oxidant defense mechanisms (GSH), resulting in

local oxidative stress possibly through formation of site-specific heme-associated ferryl radicals, peroxynitrite, and/or peroxynitrite-derived ·OH, which eventually will lead to cell death. In this respect, a study of the role of DA oxidation products (quinones) might be of value to assess the reducing capacity of the neuron.

Table I: *Oxidative stress phenomena in relation to idiopathic Parkinson's disease and three representative models of Parkinsonism induced by chemicals*

Oxidative stress phenomena	**Idiopatic Parkinson's disease**	**Manganese**	**MPTP**	**6-Hydroxydopamine**
GSH-metabolism defects	GSH ↓, γ-glutamyl-transpeptidase ↑, and mild GSSG ↑ only in SN[1,2,3,4]	GSH ↓ (mito > cyto), Gpx and GSSG-reductase activ. ↓, γ-glutamyl-transpeptidase act. ↑ in ST[5]	GSH ↓ only in midbrain[6,7,8]	GSH ↓ in ST and SN, slightly Gpx act. ↓ in SN[9]
SOD/catalase changes	MnSOD ↑ and ↔ CuZnSOD in SN[10], CuZnSOD ↑ in SN[11]	No reports	No reports	SOD and catalase ↓ in ST and SN[9]
Mitochondrial respiration (chain) defects	Complex I deficiency in SN[12,13,14,15]	Oxidative phosphory-lation[16] ↓, Complex V (or I) defect[16], or cytochromes[17] ↓, ATP ↓ and lactate[18] ↑	Irreversible Complex I inhibition in SN[19,20,21,22]	No reports
Mitochondrial P-450	No reports	Cytochrome P-450 act. and content[17] ↑ (mito > microsomes)	Protection by cyt. P-450 inhibitors, potentiation by cyt. P-450 inducers[21]	No reports

Iron accumulation/ aberrant metabolism	Total iron ↑ in SN[23,24] ↓ in GP[24], shift FeII→III[23,26], ferritin[25] ↓ and ↑[26], transferrin receptor density ↓ in putamen[27], iron in Lewy bodies[28] and neuromelanin[29]	↔ iron in ST and no protection by iron chelation[30], binding to transferrin and ferritin[31], possible axonal transport via iron pathways[32]	Total iron ↑ in SN compacta: dopamine and glial cells[33], transferrin receptor density ↓ in ST[31]	*In vitro* iron release from ferritin[34,35], attenuation by iron chelation[36]
Dopamine autoxidation	5-S-cysteinyl-dopamine/dopamine ratio ↑ in SN[37]	*In vitro* DA-quinones & oxyradicals[38a], no DA autoxidation by oxygen: no oxy-radicals[38b], *in vivo* no role of dopamine[39]	No role of dopamine[40]	*In vitro* autoxidation of 6-OHDA itself[38]
Glutamate-mediated oxidative stress	Partial protection by glutamate receptor antagonists[41]	Partial protection by glutamate receptor antagonists[41]	Partial protection by glutamate receptor antagonists[41]	Partial protection by Glu-receptor antagonists[41]
Lipid peroxidation	In SN[50]	Not *in vivo*[51], *in vitro* MPP^+ stimulates[52], MPTP inhibits[52,53]	Inhibits both *in vitro*[54,55] and *in vivo*[56,57]	*In vitro*[34]
DNA/protein/carbon-hydrate oxidations	No reports	DNA and RNA content[58] ↓	No reports	No reports

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